Methylation Inhibitor Compounds

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims benefit of U.S. patent application 60/547,902, filed February 25, 2004, the contents of which are incorporated by reference in their entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

Research supporting this application was carried out by the United States of America as represented by the Secretary, Department of Health and Human Services.

BACKGROUND OF THE INVENTION

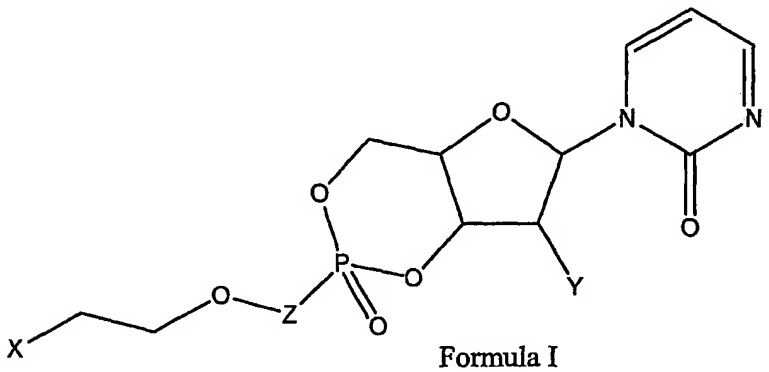
Aberrant de novo methylation of promotor regions of regulatory genes is commonly associated with cancer. Several studies have shown that de novo methylation of CpG islands in regulatory sequences of tumor suppressor genes can result in their silencing. Thus, DNA methylation may lead to abnormal growth of cancer cells. Examples of regulatory genes that are commonly hypermethylated in cancer cells include RB1 gene in retinoblastomas, the VHL gene in sporadic renal cell carcinomas, the H19 gene in Wilms' tumors, the p15 gene in leukemias, and the p16 gene in several human cancer cell lines. Thus, modulation of the hypermethylation of genes involved in the control of cell proliferation by use of methylation inhibitors has been one strategy invoked for cancer therapy, and more specifically, the use of DNA methylation inhibitors to reactivate antiproliferative, apoptotic, and differentiation-inducing genes in cancer cells.

fluorodeoxycytidine) the compounds typically suffer from one or more characteristics that detract from their use as therapeutic agents, including chemical instability (including in neutral solution), weak potency, short half-life, and generation of toxic metabolites. Thus, there still exists a need for effective, stable, and minimally toxic DNA methylation inhibitor compounds.

SUMMARY OF THE INVENTION

Compounds and compositions having those compounds are provided herein, as well as methods of making and using them. The compounds and compositions herein are useful in treatment or modulation of disease, disease symptoms or conditions in a subject. The compounds are useful as DNA methylation inhibitor compounds (and methods thereof). The compounds, compositions, and methods thereof are useful in treatment or modulation of cancer, cancer symptoms or cancer conditions in a subject.

One aspect is an isolated compound of Formula I:



Formula I

wherein,

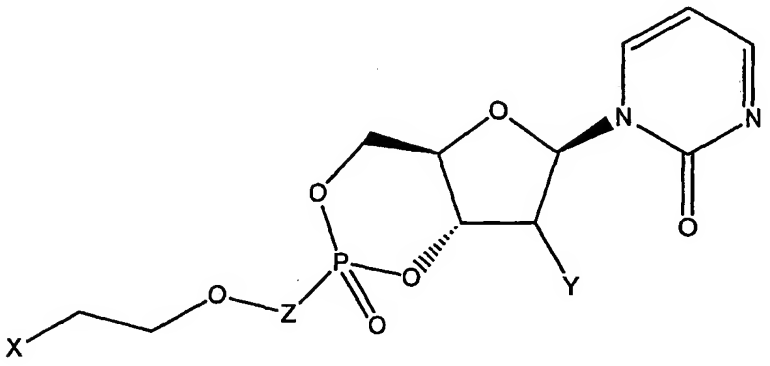
each X is independently NR^1R^2, or $NR^1R^2R^{3+}$;

each R^1, R^2 and R^3 is independently H or alkyl;

each Y is independently H, OH, or halogen; and

each Z is independently a bond or –P(O)(OH)-O-;

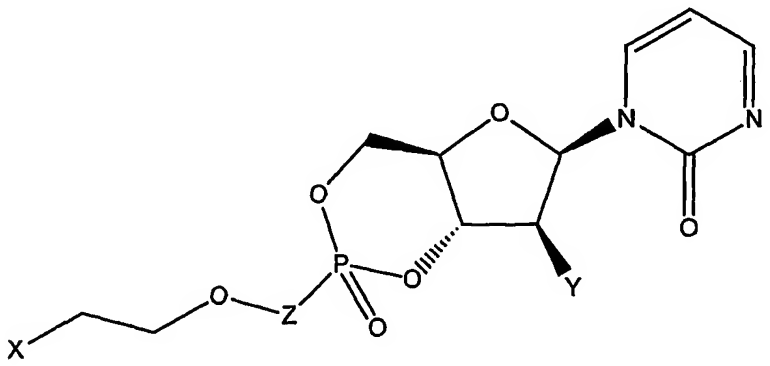
Another aspect is the isolated compound of Formula II or pharmaceutically acceptable salt or hydrate thereof:



Formula II

wherein X, R^1, R^2, R^3, Y and Z are as defined above.

Another aspect is the isolated compound of Formula III or pharmaceutically acceptable salt or hydrate thereof,

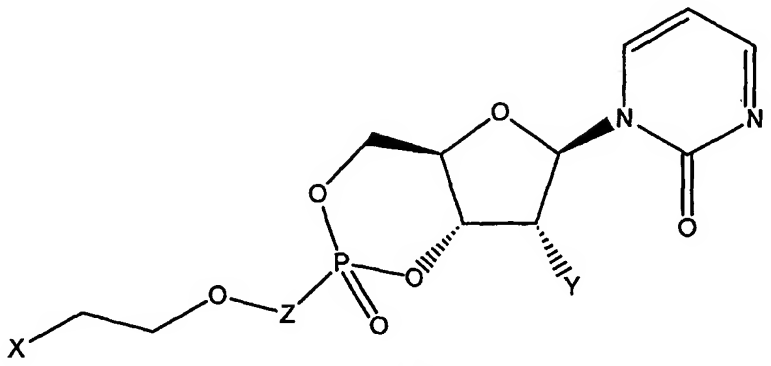


Formula III

wherein X, R^1, R^2, R^3, Y and Z are as defined above.

formulae herein, wherein X is NH_2;. X is $(NMe_3)^+$; and the compounds as delineated in Tables I and II herein.

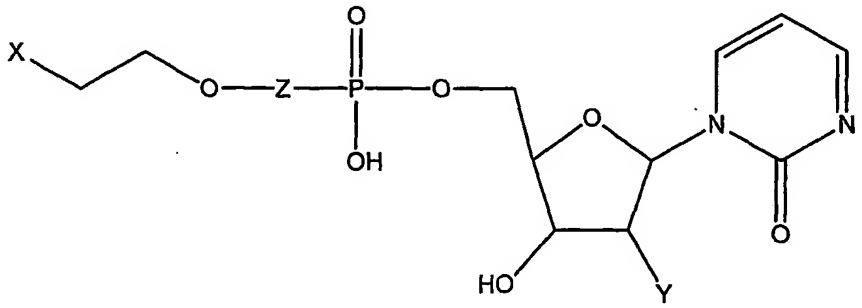
Another aspect is the isolated compound of Formula IV or pharmaceutically acceptable salt or hydrate thereof:



Formula IV

wherein X, R^1; R^2, R^3 and Z are as defined above and Y is OH.

Another aspect is the isolated compound of Formula V:



Formula V

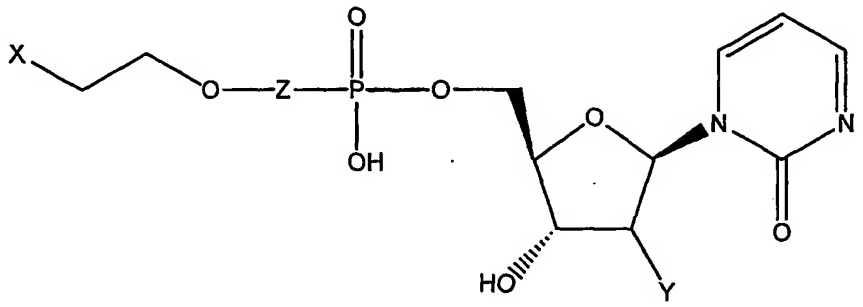
wherein,

each X is independently NR^1R^2, or $NR^1R^2R^{3+}$;

each Z is independently a bond or –P(O)(OH)-O-;

or pharmaceutically acceptable salt or hydrate thereof.

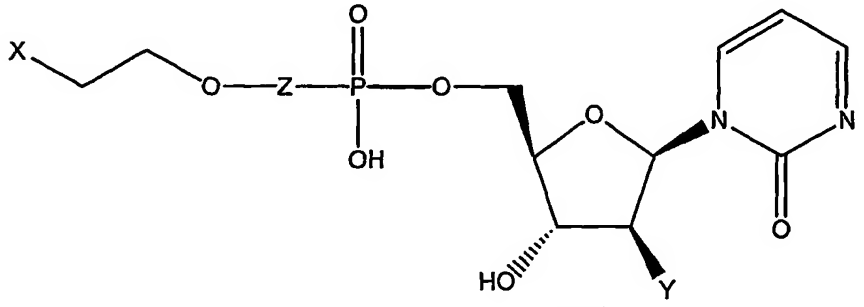
Another aspect is the isolated compound of Formula VI or pharmaceutically acceptable salt or hydrate thereof:



Formula VI

wherein X, R^1, R^2, R^3, Y and Z are as defined above.

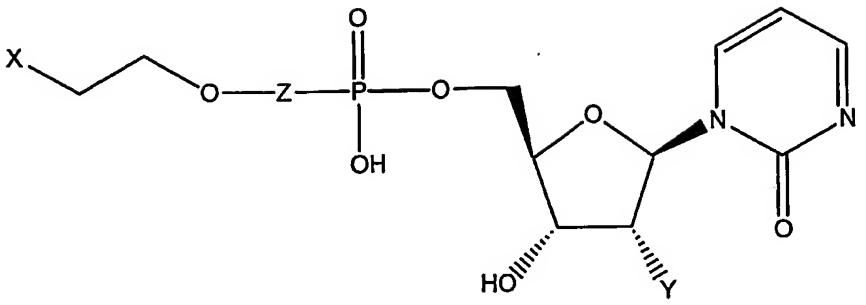
Another aspect is the isolated compound of Formula VII or pharmaceutically acceptable salt or hydrate thereof:



Formula VII

wherein X, R^1, R^2, R^3, Y and Z are as defined above.

Another aspect is the isolated compound of Formula VIII or pharmaceutically acceptable salt or hydrate thereof:



Formula VIII

wherein X, R^1; R^2, R^3 and Z are as defined above and Y is OH.

Other aspects are compounds of the formulae herein, wherein Y is halogen; wherein Y is fluoro; wherein Y is hydrogen. Other aspects are compounds of the formulae herein, wherein X is NH_2;. X is $(NMe_3)^+$.

In another aspect, the invention relates to a composition having a therapeutically effective amount of a compound (or pharmaceutically acceptable salt or hydrate thereof) according to any of the formulae herein and a pharmaceutically acceptable carrier. The composition can further have an additional therapeutic agent, the additional agent can be an anticancer agent.

Another aspect is a method of treating a DNA methyl transferase (DNMT) mediated disease, disease symptom or condition that includes administration to a subject in need of such treatment a compound (or pharmaceutically acceptable salt or hydrate thereof) according to any of the formulae herein, or composition thereof. The disease, disease symptom or condition can involve hypermethylation of DNA; the administration can be by oral administration.

Another aspect is a method of assessing the effect of a test compound on methylation of DNA in a cell including: (i) contacting a test compound with a cell that

methylation; and measuring the methylation of DNA in the cell; and (iii) comparing the results of step (i) with the results of step (ii). In these methods, the cell can include a hypermethylated nucleic acid molecule; a CpG dinucleotide; and can be a mammalian tumor cell.

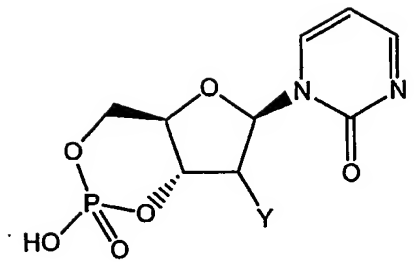
Another aspect is a method of reversing DNA methylation in a cell, including administering to a cell a therapeutically effective amount of a compound of any of the formulae herein. The cell can be in a subject or in vitro.

The invention also relates to a method of treating cancer in a subject including administering an effective amount of a compound according to any of the formulae herein. The cancer can be ovarian, breast, colon, rectal, lung, prostate, pancreatic, bladder, solid tumor, or any tumor having a silenced tumor suppressor gene. The cancer can be any associated with or exemplified by cancer cell lines, including for example, T24, HCT15, CFPAC-1, SW48, HT-29, PC3, or CALU-1. The method can further include administration of an additional anticancer agent; an anti-nausea or an anti-anemia agent. The administration of the additional agent(s) can be concurrently or sequentially, and can be individually or in a combined formulation.

The invention also relates to a kit having a compound of any of the formulae herein and instructions for in vivo or in vitro use of the compound. The in vitro use can be screening for demethylation of a hypermethylated DNA.

The invention also relates to a kit having a compound of any of the formulae herein and instructions for administration to a subject. The subject can be in need of treatment for a hypermethylated DNA mediated disease, disease symptom or condition; in need of treatment for a hyperproliferative disease, disease symptom or condition; in need of treatment for cancer. The subject can be a human; or a rat or mouse. The administration can be oral;or intravenous or intraperitoneal.

Another aspect is a method of making a compound of any of the formulae herein, comprising converting a compound of Formula B, wherein Y is H, OH, O-PG, or halo; and PG is a protecting group:



Formula B

to a compound of Formula I herein. The process can further include converting the compound of Formula B to the corresponding diphosphate; and further include removal of an oxygen- or nitrogen-protecting group.

In one aspect, a method of making any of the compounds delineated herein involves one or more reactions and/or reagents as delineated herein.

In other aspects, the invention relates to a composition comprising a compound of any of the formulae herein, an additional therapeutic agent, and a pharmaceutically acceptable carrier. The additional therapeutic agent can be an anticancer agent (e.g., arabinofuranosyl cytosine (ara-C), 5-fluorouracil (5-FU) and taxol). The additional therapeutic agent can also be a histone deacetylase inhibitor: See, e.g., Lemaire et al. *Leukemia Lymphoma* 2004, 45, 147-154; Leone et al. *Clin Immunol.* 2003, 109, 89-102; Shaker et al. *Leukemia Res* 2003, 27, 437-444; Primeau et al. *Int. J. Cancer* 2003, 103, 177-184.

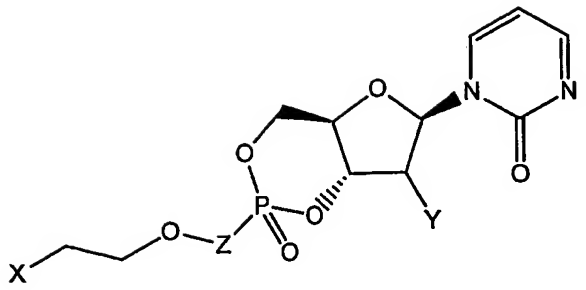
Yet another aspect of this invention relates to a method of treating a subject (e.g., mammal, human, horse, dog, cat) having a DNA-methylation-mediated disease or disease symptom (including, but not limited to cancer). The method includes administering to the subject (including a subject identified as in need of such treatment) an effective amount of a compound described herein, or a composition described herein to produce such effect. Identifying a subject in need of such

The invention also relates to a method of making a compound described herein. Alternatively, the method includes taking any one of the intermediate compounds described herein and reacting it with one or more chemical reagents in one or more steps to produce a compound described herein.

In other aspects the invention relates to a compound made by a process that includes any one, or more, of the reactions delineated herein. In particular, the reactions in the general schemes and examples herein. In other aspects, the process includes the reagent or reagents, or reaction conditions delineated herein.

Also within the scope of this invention is a packaged product. The packaged product includes a container, one of the aforementioned compounds in the container, and a legend (e.g., a label or an insert) associated with the container and indicating administration of the compound for treating a disorder associated with DNA-methylation modulation.

In other embodiments, the compounds, compositions, and methods delineated herein are any of the compounds of Table I or Table II (or salts or solvates thereof) herein or methods including them.



Compound	X	Y	Z
1	NH_2	H	bond
2	NH_2	F	bond
3	NH_2	OH	bond
4	NH_2	OBz	bond
5	$N(CH_3)_3^+$	H	bond
6	$N(CH_3)_3^+$	F	bond
7	$N(CH_3)_3^+$	OH	bond
8	$N(CH_3)_3^+$	OBz	bond
9	NH_2	H	-P(O)(OH)-O-
10	NH_2	F	-P(O)(OH)-O-
11	NH_2	OH	-P(O)(OH)-O-
12	NH_2	OBz	-P(O)(OH)-O-
13	$N(CH_3)_3^+$	H	-P(O)(OH)-O-
14	$N(CH_3)_3^+$	F	-P(O)(OH)-O-
15	$N(CH_3)_3^+$	OH	-P(O)(OH)-O-
16	$N(CH_3)_3^+$	OBz	-P(O)(OH)-O-

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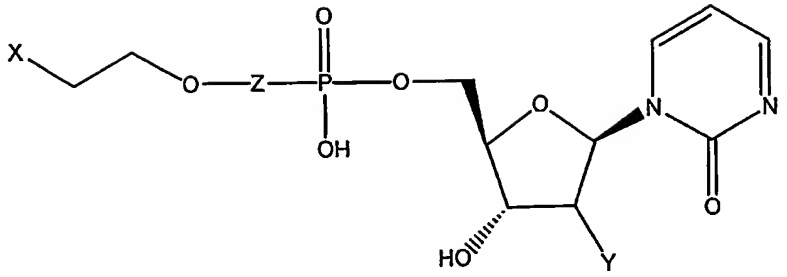

Compound	X	Y	Z
17	NH_2	H	bond
18	NH_2	F	bond
19	NH_2	OH	bond
20	NH_2	OBz	bond
21	$N(CH_3)_3^+$	H	bond
22	$N(CH_3)_3^+$	F	bond
23	$N(CH_3)_3^+$	OH	bond
24	$N(CH_3)_3^+$	OBz	bond
25	NH_2	H	-P(O)(OH)-O-
26	NH_2	F	-P(O)(OH)-O-
27	NH_2	OH	-P(O)(OH)-O-
28	NH_2	OBz	-P(O)(OH)-O-
29	$N(CH_3)_3^+$	H	-P(O)(OH)-O-
30	$N(CH_3)_3^+$	F	-P(O)(OH)-O-
31	$N(CH_3)_3^+$	OH	-P(O)(OH)-O-
32	$N(CH_3)_3^+$	OBz	-P(O)(OH)-O-

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accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and from the claims.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 shows the structures of zebularine and cytidine.

FIG. 2 illustrates the effects of 5-azacytidine (5-azaC) and zebularine exposure to T24 cell proliferation

FIG. 3 illustrates radiolabeled metabolites of zebularine incubated with T-24 cells.

FIG. 4 illustrates the enzymatic characterization of zebularine metabolites.

FIG. 5 illustrates HPLC radiochromatograms of zebularine metabolites.

FIG. 6 illustrates formation of phosphorylated zebularine metabolites.

FIG. 7 profiles zebularine metabolite levels in T-24 cells.

FIG. 8 illustrates incorporation of zebularine into DNA and RNA of T-24 cells.

FIG. 9 illustrates the HPLC radiochromatograms of zebularine metabolites *in vivo* in nude mice in tumor and in normal muscle.

FIG. 10 illustrates the effect of zebularine on human bladder carcinoma cell proliferation.

FIG. 11 illustrates a metabolic activation pathway for zebularine.

DETAILED DESCRIPTION OF THE INVENTION

The terms "halo" and "halogen" refer to any radical of fluorine, chlorine, bromine or iodine. The term "alkyl" refers to a hydrocarbon chain that may be a straight chain or branched chain, containing the indicated number of carbon atoms. For example, C1-C10 indicates that the group may have from 1 to 10 (inclusive) carbon atoms in it. The term "lower alkyl" refers to a C1-C6 alkyl chain. The term "alkenyl" refers to an unsaturated hydrocarbon chain that may be a straight chain or branched chain, containing the indicated number of carbon atoms. The term "alkynyl" refers to an unsaturated hydrocarbon chain that may be a straight chain or

"ester" refers to a C(O)O-alkyl or C(O)O-aryl group. An "amido" is an $C(O)NH_2$, an "N-alkyl-substitited amido" is of the formula C(O)N(H)(alkyl).

The term "cycloalkyl" refers to a 6-carbon monocyclic or 10-carbon bicyclic nonaromatic ring system wherein 0, 1, 2, 3, or 4 atoms of each ring may be substituted by a substituent. Examples of cycloalkyl groups include cyclopentyl, cyclohexyl, cyclohexenyl, bicyclo[2.2.1]hept-2-enyl, dihydronaphthalenyl, benzocyclopentyl, and the like.

The term "aryl" refers to a 6-carbon monocyclic or 10-carbon bicyclic aromatic ring system wherein 0, 1, 2, 3, or 4 atoms of each ring may be substituted by a substituent. Examples of aryl groups include phenyl, naphthyl and the like. The term "arylalkyl" or the term "aralkyl" refers to alkyl substituted with an aryl. The term "arylalkoxy" refers to an alkoxy substituted with aryl.

The term "heteroaryl" refers to an aromatic 5-8 membered monocyclic, 8-12 membered bicyclic, or 11-14 membered tricyclic ring system having 1-3 heteroatoms if monocyclic, 1-6 heteroatoms if bicyclic, or 1-9 heteroatoms if tricyclic, said heteroatoms selected from O, N, or S (e.g., carbon atoms and 1-3, 1-6, or 1-9 heteroatoms of N, O, or S if monocyclic, bicyclic, or tricyclic, respectively), wherein 0, 1, 2, 3, or 4 atoms of each ring may be substituted by a substituent. Examples of heteroaryl groups include pyridyl, furyl or furanyl, imidazolyl, benzimidazolyl, thienyl, indolyl, thiazolyl, and the like. The term "heteroarylalkyl" or the term "heteroaralkyl" refers to an alkyl substituted with a heteroaryl. The term "heteroarylalkoxy" refers to an alkoxy substituted with heteroaryl.

The term "heterocyclyl" refers to a nonaromatic 5-8 membered monocyclic, 8-12 membered bicyclic, or 11-14 membered tricyclic ring system comprising 1-3 heteroatoms if monocyclic, 1-6 heteroatoms if bicyclic, or 1-9 heteroatoms if tricyclic, said heteroatoms selected from O, N, or S (e.g., carbon atoms and 1-3, 1-6, or 1-9 heteroatoms of N, O, or S if monocyclic, bicyclic, or tricyclic, respectively), wherein 0, 1, 2, 3, or 4 atoms of each ring may be substituted by a substituent. Examples of heterocyclyl groups include tetrahydrofuryl, piperidinyl, pyrrolidinyl, morpholinyl, dihydrothiophenyl, dihydrobenzothiophenyl, indolinyl, and the like.

the group hydroxy, mercapto, amino, alkoxy, carboxylic acid, ester, amido, N-alkyl-substitited amido, halo, nitro, and nitrile; or 1 to 4 independent NR^6R^6, $C(O)NR^6R^6$, OR^6, SR^6, $C(O)OR^6$, $C(O)R^6$, $S(O)_nR^6$, NO_2, CN, halo, $NR^6C(O)R^6$, or $NR^6S(O)_nR^6$; wherein n is 1 or 2; and each R^6 is independently alkyl, alkenyl, aryl, arylalkyl, or heteroarylalkyl, each optionally substituted with 1-4 independent substituents selected from the group hydroxy, mercapto, amino, alkoxy, carboxylic acid, ester, amido, N-alkyl-substitited amido, halo, nitro, and nitrile.

Combinations of substituents and variables envisioned by this invention are only those that result in the formation of stable compounds. The term "stable", as used herein, refers to compounds which possess stability sufficient to allow manufacture and which maintains the integrity of the compound for a sufficient period of time to be useful for the purposes detailed herein (e.g., therapeutic formulations, reagents, kits). The compounds produced by the methods herein can be incorporated into compositions, including pills, capsules, gel caps, solutions, tablets, crèmes, or ointments for administration to a subject (e.g., human, animal).

Acids and bases useful in the methods herein are known in the art. Acid catalysts are any acidic chemical, which can be inorganic (e.g., hydrochloric, sulfuric, nitric acids) or organic (e.g., camphorsulfonic acid, p-toluenesulfonic acid, acetic acid) in nature. Acids are useful in either catalytic or stoichiometric amounts to facilitate chemical reactions. Bases are any basic chemical, which can be inorganic (e.g., sodium bicarbonate, potassium hydroxide) or organic (e.g., triethylamine, pyridine) in nature. Bases are useful in either catalytic or stoichiometric amounts to facilitate chemical reactions.

Alkylating agents are any reagent that is capable of effecting the alkylation of the functional group at issue (e.g., oxygen atom of an alcohol, nitrogen atom of an amino group). Alkylating agents are known in the art, including in the references cited herein, and include alkyl halides (e.g., methyl iodide, benzyl bromide or chloride), alkyl sulfates (e.g., methyl sulfate), or other alkyl group-leaving group combinations known in the art. Leaving groups are any stable species that can detach from a molecule during a reaction (e.g., elimination reaction, substitution reaction) and are known in the art, including in the references cited herein, and include halides

carbamates (e.g., N(Me)C(O)Ot-Bu), phosphonates (e.g., -OP(O)(OEt)$_2$), water or alcohols (protic conditions), and the like.

Nucleophilic agents are known in the art and are described in the chemical texts and treatises referred to herein. The chemicals used in the aforementioned methods may include, for example, solvents, reagents, catalysts, protecting group and deprotecting group reagents and the like. The methods described above may also additionally comprise steps, either before or after the steps described specifically herein, to add or remove suitable protecting groups in order to ultimately allow synthesis of the compound of the formulae described herein. The methods delineated herein contemplate converting compounds of one formula to compounds of another formula. The process of converting refers to one or more chemical transformations, which can be performed *in situ*, or with isolation of intermediate compounds. The transformations can include reacting the starting compounds or intermediates with additional reagents using techniques and protocols known in the art, including those in the references cited herein. Intermediates can be used with or without purification (e.g., filtration, distillation, crystallization, chromatography).

The compounds delineated herein can be synthesized using conventional methods, as illustrated generally in the schemes herein.

In the structures in Schemes I and II, Y, X and Z are as defined in the formulae herein, and PG is a protecting group (e.g., an oxygen-protecting group, a nitrogen-protecting group).

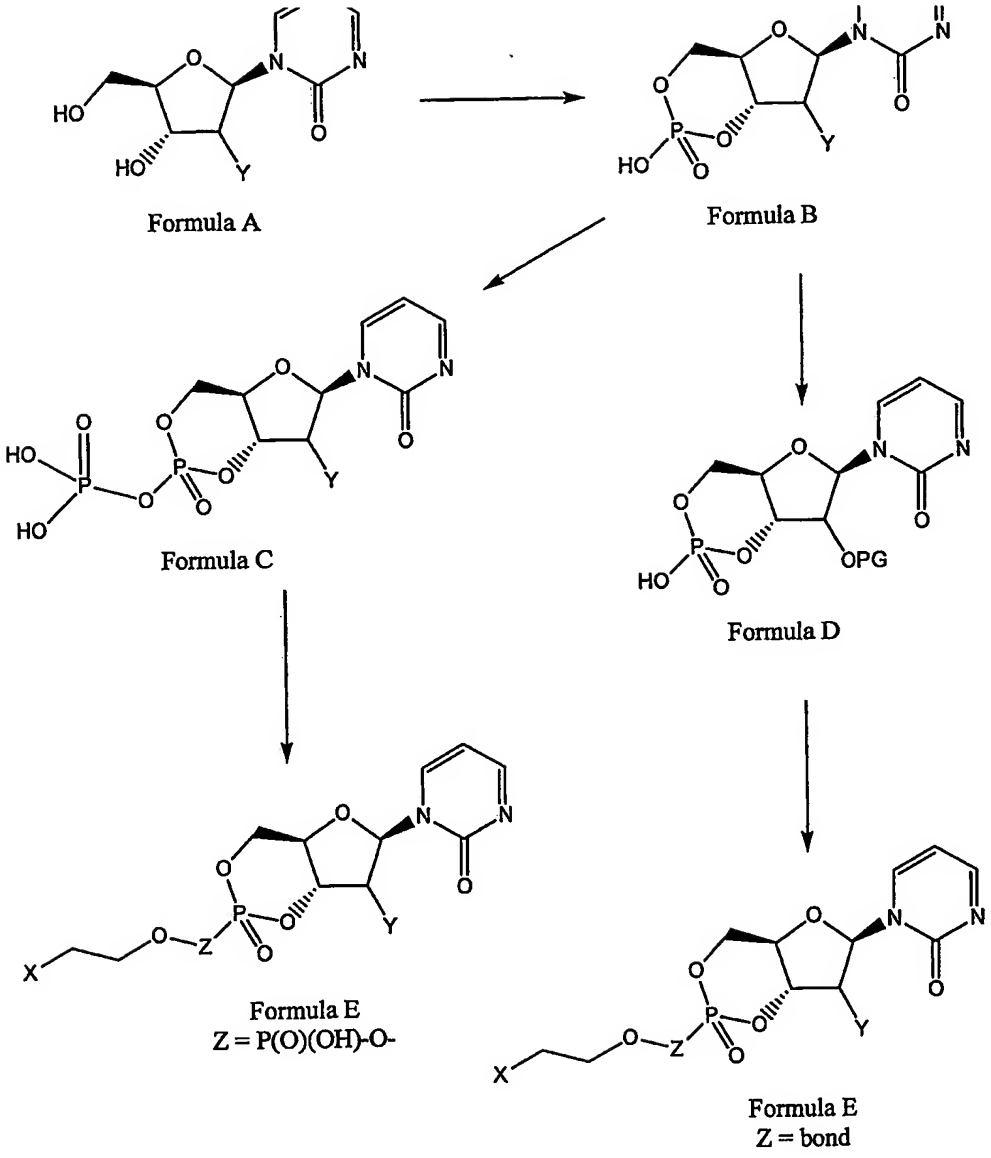
The term "protecting group," as used herein, refers to a labile chemical moiety which is known in the art to protect a chemical group (e.g., oxygen- or nitrogen-containing moiety) against undesired reactions during synthetic procedures. After said synthetic procedure(s) the protecting group as described herein may be selectively removed. Protecting groups as known in the art are described generally in T.H. Greene and P.G. M. Wuts, Protective Groups in Organic Synthesis, 3rd edition, John Wiley & Sons, New York (1999).

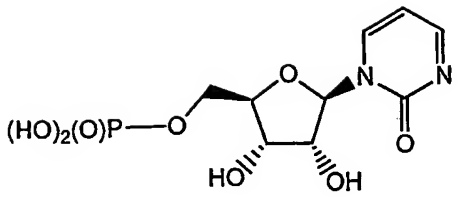
The term "oxygen protecting group," as used herein, refers to a labile chemical moiety which is known in the art to protect a hydroxyl group against undesired reactions during synthetic procedures. After said synthetic procedure(s) the hydroxy

Protective Groups in Organic Synthesis, 3rd edition, John Wiley & Sons, New York (1999). Examples of hydroxyl protecting groups include acetyl (Ac or -C(O)CH_3), benzoyl (Bz or -C(O)C_6H_5), benzyl (Bn), t-butyldimethylsilyl, (TBDMS), and trimethylsilyl (TMS or-Si$(CH_3)_3$).

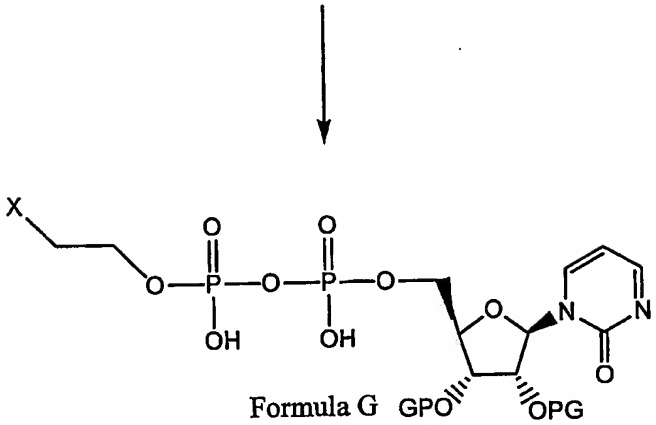
The term "nitrogen protecting group," as used herein, refers to a labile chemical moiety which is known in the art to protect a nitrogen-containing group against undesired reactions during synthetic procedures. After said synthetic procedure(s) the nitrogen protecting group as described herein may be selectively removed. Nitrogen protecting groups as known in the are described generally in T.H. Greene and P.G. M. Wuts, Protective Groups in Organic Synthesis, 3rd edition, John Wiley & Sons, New York (1999). Examples of nitrogen protecting groups include acetyl (Ac or -C(O)CH_3), phthalimido, BOC (t-butoxycarbonyl), and the like.

Compounds of Formula A can be converted to compounds of Formula B using standard cyclization conditions such as those essentially described by Geniser et al. (*Synthesis*, 53-54 (1989)). In instances where Y in Formula B is hydroxy, the hydroxy group can be protected (e.g., benzoyl) to give compounds of Formula B wherein Y is a protected hydroxy group (OPG). Compounds of Formula B can then be converted to the diphosphate compounds of Formula C under standard conditions (e.g., DCC, $(t\text{-}Bu_3NO)_3PO$) and then coupled (DCC and choline chloride or N-protected ethanolamine, such as phthalimido-protected ethanolamine) followed by deprotection, if appropriate (e.g., removal of protecting groups, hydrolysis, aqueous acid, ammonia/MeOH, hydrazine), to give compounds of Formula E where Z is -P(O)(OH)-O-. Compounds of Formula B where Y is H or halogen can also be directly coupled (DCC and choline chloride or N-protected ethanolamine, such as phthalimido-protected ethanolamine) followed by deprotection, if appropriate (e.g., removal of protecting groups, hydrolysis, aqueous acid, ammonia/MeOH, hydrazine), to give compounds of Formula E where Z is a bond. For compounds of Formula B where Y is hydroxy, the hydroxy group is protected to give a compound of Formula D, which is coupled and then further deprotected to give compounds of Formula E where Z is a bond.

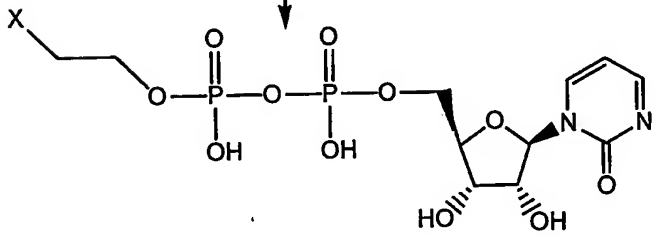

Formula A
Formula B
Formula C
Formula D
Formula E
Z = P(O)(OH)-O-
Formula E
Z = bond



Formula F



Formula G



Formula H

Scheme II illustrates general synthesis of compounds delineated herein. Compounds of Formula F can be protected with oxygen protecting groups then coupled (DCC and phosphorylcholine chloride or N-protected ethanolaminephosphoric acid ester derivative, such as phthalimido-protected ethanolaminephosphoric acid ester) followed by deprotection, if appropriate (e.g., removal of protecting groups, hydrolysis, aqueous acid, ammonia/MeOH, hydrazine),

if appropriate (e.g., removal of protecting groups, hydrolysis, aqueous acid, ammonia/MeOH, hydrazine), to give compounds of Formula H.

As can be appreciated by the skilled artisan, the synthetic schemes herein are not intended to comprise a comprehensive list of all means by which the compounds described and claimed in this application may be synthesized. Further methods will be evident to those of ordinary skill in the art. Additionally, the various synthetic steps described above may be performed in an alternate sequence or order to give the desired compounds. Synthetic chemistry transformations and protecting group methodologies (protection and deprotection) useful in synthesizing the compounds described herein are known in the art and include, for example, those such as described in R. Larock, Comprehensive Organic Transformations, VCH Publishers (1989); T.W. Greene and P.G.M. Wuts, Protective Groups in Organic Synthesis, 2d. Ed., John Wiley and Sons (1991); L. Fieser and M. Fieser, Fieser and Fieser's Reagents for Organic Synthesis, John Wiley and Sons (1994); and L. Paquette, ed., Encyclopedia of Reagents for Organic Synthesis, John Wiley and Sons (1995) and subsequent editions thereof.

The term "treating" or "treated" refers to administering a compound described herein to a subject with the purpose to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve, or affect a disease, the symptoms of the disease or the predisposition toward the disease.

"An effective amount" refers to an amount of a compound, which confers a therapeutic effect on the treated subject. The therapeutic effect may be objective (i.e., measurable by some test or marker) or subjective (i.e., subject gives an indication of or feels an effect). An effective amount of the compound described above may range from about 50 mg/Kg to about 200 mg/Kg. Effective doses will also vary depending on route of administration, as well as the possibility of co-usage with other agents.

DNA methylation-modulating compounds can be identified through both *in vitro* (e.g., cell and non-cell based) and *in vivo* methods. Representative examples of these methods are described in detail in the Examples.

Combinations of substituents and variables envisioned by this invention are only those that result in the formation of stable compounds. The term "stable", as used

be useful for the purposes detailed herein (e.g., therapeutic or prophylactic administration to a subject).

The compounds of this invention may contain one or more asymmetric centers and thus occur as racemates and racemic mixtures, single enantiomers, individual diastereomers and diastereomeric mixtures. All such isomeric forms of these compounds are expressly included in the present invention. The compounds of this invention may also be represented in multiple tautomeric forms, in such instances, the invention expressly includes all tautomeric forms of the compounds described herein (e.g., alkylation of a ring system may result in alkylation at multiple sites, the invention expressly includes all such reaction products). All such isomeric forms of such compounds are expressly included in the present invention. All crystal forms of the compounds described herein are expressly included in the present invention.

As used herein, the compounds of this invention, including the compounds of formulae described herein, are defined to include pharmaceutically acceptable derivatives or prodrugs thereof. A "pharmaceutically acceptable derivative or prodrug" means any pharmaceutically acceptable salt, ester, salt of an ester, or other derivative of a compound of this invention which, upon administration to a recipient, is capable of providing (directly or indirectly) a compound of this invention. Particularly favored derivatives and prodrugs are those that increase the bioavailability of the compounds of this invention when such compounds are administered to a mammal (e.g., by allowing an orally administered compound to be more readily absorbed into the blood) or which enhance delivery of the parent compound to a biological compartment (e.g., the brain or lymphatic system) relative to the parent species. Preferred prodrugs include derivatives where a group which enhances aqueous solubility or active transport through the gut membrane is appended to the structure of formulae described herein.

The compounds of this invention may be modified by appending appropriate functionalities to enhance selective biological properties. Such modifications are known in the art and include those which increase biological penetration into a given biological compartment (e.g., blood, lymphatic system, central nervous system), increase oral availability, increase solubility to allow administration by injection, alter

those derived from pharmaceutically acceptable inorganic and organic acids and bases. Examples of suitable acid salts include acetate, adipate, alginate, aspartate, benzoate, benzenesulfonate, bisulfate, butyrate, citrate, camphorate, camphorsulfonate, digluconate, dodecylsulfate, ethanesulfonate, formate, fumarate, glucoheptanoate, glycolate, hemisulfate, heptanoate, hexanoate, hydrochloride, hydrobromide, hydroiodide, 2-hydroxyethanesulfonate, lactate, maleate, malonate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, nitrate, palmoate, pectinate, persulfate, 3-phenylpropionate, phosphate, picrate, pivalate, propionate, salicylate, succinate, sulfate, tartrate, thiocyanate, tosylate and undecanoate. Other acids, such as oxalic, while not in themselves pharmaceutically acceptable, may be employed in the preparation of salts useful as intermediates in obtaining the compounds of the invention and their pharmaceutically acceptable acid addition salts. Salts derived from appropriate bases include alkali metal (e.g., sodium), alkaline earth metal (e.g., magnesium), ammonium and N-$(alkyl)_4^+$ salts. This invention also envisions the quaternization of any basic nitrogen-containing groups of the compounds disclosed herein. Water or oil-soluble or dispersible products may be obtained by such quaternization.

The compositions delineated herein include the compounds of the formulae delineated herein, as well as additional therapeutic agents if present, in amounts effective for achieving a modulation of disease or disease symptoms, including ion channel-mediated disorders or symptoms thereof.

The term "pharmaceutically acceptable carrier or adjuvant" refers to a carrier or adjuvant that may be administered to a patient, together with a compound of this invention, and which does not destroy the pharmacological activity thereof and is nontoxic when administered in doses sufficient to deliver a therapeutic amount of the compound.

Pharmaceutically acceptable carriers, adjuvants and vehicles that may be used in the pharmaceutical compositions of this invention include, but are not limited to, ion exchangers, alumina, aluminum stearate, lecithin, self-emulsifying drug delivery systems (SEDDS) such as d-α-tocopherol polyethyleneglycol 1000 succinate,

substances such as phosphates, glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water, salts or electrolytes, such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, zinc salts, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulose-based substances, polyethylene glycol, sodium carboxymethylcellulose, polyacrylates, waxes, polyethylene-polyoxypropylene-block polymers, polyethylene glycol and wool fat. Cyclodextrins such as α-, β-, and γ-cyclodextrin, or chemically modified derivatives such as hydroxyalkylcyclodextrins, including 2- and 3-hydroxypropyl-β-cyclodextrins, or other solubilized derivatives may also be advantageously used to enhance delivery of compounds of the formulae described herein.

The pharmaceutical compositions of this invention may be administered orally, parenterally, by inhalation spray, topically, rectally, nasally, buccally, vaginally or via an implanted reservoir, preferably by oral administration or administration by injection. The pharmaceutical compositions of this invention may contain any conventional non-toxic pharmaceutically-acceptable carriers, adjuvants or vehicles. In some cases, the pH of the formulation may be adjusted with pharmaceutically acceptable acids, bases or buffers to enhance the stability of the formulated compound or its delivery form. The term parenteral as used herein includes subcutaneous, intracutaneous, intravenous, intramuscular, intraarticular, intraarterial, intrasynovial, intrasternal, intrathecal, intralesional and intracranial injection or infusion techniques.

The pharmaceutical compositions may be in the form of a sterile injectable preparation, for example, as a sterile injectable aqueous or oleaginous suspension. This suspension may be formulated according to techniques known in the art using suitable dispersing or wetting agents (such as, for example, Tween 80) and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally acceptable diluent or solvent, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are mannitol, water, Ringer's solution and isotonic sodium

employed including synthetic mono- or diglycerides. Fatty acids, such as oleic acid and its glyceride derivatives are useful in the preparation of injectables, as are natural pharmaceutically-acceptable oils, such as olive oil or castor oil, especially in their polyoxyethylated versions. These oil solutions or suspensions may also contain a long-chain alcohol diluent or dispersant, or carboxymethyl cellulose or similar dispersing agents which are commonly used in the formulation of pharmaceutically acceptable dosage forms such as emulsions and or suspensions. Other commonly used surfactants such as Tweens or Spans and/or other similar emulsifying agents or bioavailability enhancers which are commonly used in the manufacture of pharmaceutically acceptable solid, liquid, or other dosage forms may also be used for the purposes of formulation.

The pharmaceutical compositions of this invention may be orally administered in any orally acceptable dosage form including, but not limited to, capsules, tablets, emulsions and aqueous suspensions, dispersions and solutions. In the case of tablets for oral use, carriers which are commonly used include lactose and corn starch. Lubricating agents, such as magnesium stearate, are also typically added. For oral administration in a capsule form, useful diluents include lactose and dried corn starch. When aqueous suspensions and/or emulsions are administered orally, the active ingredient may be suspended or dissolved in an oily phase is combined with emulsifying and/or suspending agents. If desired, certain sweetening and/or flavoring and/or coloring agents may be added.

The pharmaceutical compositions of this invention may also be administered in the form of suppositories for rectal administration. These compositions can be prepared by mixing a compound of this invention with a suitable non-irritating excipient which is solid at room temperature but liquid at the rectal temperature and therefore will melt in the rectum to release the active components. Such materials include, but are not limited to, cocoa butter, beeswax and polyethylene glycols.

Topical administration of the pharmaceutical compositions of this invention is useful when the desired treatment involves areas or organs readily accessible by topical application. For application topically to the skin, the pharmaceutical composition should be formulated with a suitable ointment containing the active

petroleum, white petroleum, propylene glycol, polyoxyethylene polyoxypropylene compound, emulsifying wax and water. Alternatively, the pharmaceutical composition can be formulated with a suitable lotion or cream containing the active compound suspended or dissolved in a carrier with suitable emulsifying agents. Suitable carriers include, but are not limited to, mineral oil, sorbitan monostearate, polysorbate 60, cetyl esters wax, cetearyl alcohol, 2-octyldodecanol, benzyl alcohol and water. The pharmaceutical compositions of this invention may also be topically applied to the lower intestinal tract by rectal suppository formulation or in a suitable enema formulation. Topically-transdermal patches are also included in this invention.

The pharmaceutical compositions of this invention may be administered by nasal aerosol or inhalation. Such compositions are prepared according to techniques well-known in the art of pharmaceutical formulation and may be prepared as solutions in saline, employing benzyl alcohol or other suitable preservatives, absorption promoters to enhance bioavailability, fluorocarbons, and/or other solubilizing or dispersing agents known in the art.

A composition having the compound of the formulae herein and an additional agent (e.g., a therapeutic agent) can be administered using an implantable device. Implantable devices and related technology are known in the art and are useful as delivery systems where a continuous, or timed-release delivery of compounds or compositions delineated herein is desired. Additionally, the implantable device delivery system is useful for targeting specific points of compound or composition delivery (e.g., localized sites, organs). Negrin et al., Biomaterials, 22(6):563 (2001). Timed-release technology involving alternate delivery methods can also be used in this invention. For example, timed-release formulations based on polymer technologies, sustained-release techniques and encapsulation techniques (e.g., polymeric, liposomal) can also be used for delivery of the compounds and compositions delineated herein.

Also within the invention is a patch to deliver active chemotherapeutic combinations herein. A patch includes a material layer (e.g., polymeric, cloth, gauze, bandage) and the compound of the formulae herein as delineated herein. One side of the material layer can have a protective layer adhered to it to resist passage of the

natural or synthetic origin, that when contacted with the skin of a subject, temporarily adheres to the skin. It can be water resistant. The adhesive can be placed on the patch to hold it in contact with the skin of the subject for an extended period of time. The adhesive can be made of a tackiness, or adhesive strength, such that it holds the device in place subject to incidental contact, however, upon an affirmative act (e.g., ripping, peeling, or other intentional removal) the adhesive gives way to the external pressure placed on the device or the adhesive itself, and allows for breaking of the adhesion contact. The adhesive can be pressure sensitive, that is, it can allow for positioning of the adhesive (and the device to be adhered to the skin) against the skin by the application of pressure (e.g., pushing, rubbing,) on the adhesive or device.

Compounds herein are administered in a dosage ranging from about 10 to about 500 mg/kg of body weight, preferably dosages between 50 mg and 200 mg/dose, every 4 to 120 hours, or according to the requirements of the particular drug. The methods herein contemplate administration of an effective amount of compound or compound composition to achieve the desired or stated effect. Typically, the pharmaceutical compositions of this invention will be administered from about 1 to about 6 times per day or alternatively, as a continuous infusion. Such administration can be used as a chronic or acute therapy. The amount of active ingredient that may be combined with the carrier materials to produce a single dosage form will vary depending upon the host treated and the particular mode of administration. A typical preparation will contain from about 5% to about 95% active compound (w/w). Alternatively, such preparations contain from about 20% to about 80% active compound.

Lower or higher doses than those recited above may be required. Specific dosage and treatment regimens for any particular patient will depend upon a variety of factors, including the activity of the specific compound employed, the age, body weight, general health status, sex, diet, time of administration, rate of excretion, drug combination, the severity and course of the disease, condition or symptoms, the patient's disposition to the disease, condition or symptoms, and the judgment of the treating physician.

necessary. Subsequently, the dosage or frequency of administration, or both, may be reduced, as a function of the symptoms, to a level at which the improved condition is retained when the symptoms have been alleviated to the desired level, treatment should cease. Patients may, however, require intermittent treatment on a long-term basis upon any recurrence of disease symptoms.

When the compositions of this invention comprise a combination of a compound of the formulae described herein and one or more additional therapeutic or prophylactic agents, both the compound and the additional agent should be present at dosage levels of between about 1 to 100%, and more preferably between about 5 to 95% of the dosage normally administered in a monotherapy regimen. The additional agents may be administered separately, as part of a multiple dose regimen, from the compounds of this invention. Alternatively, those agents may be part of a single dosage form, mixed together with the compounds of this invention in a single composition.

"Prodrug", as used herein means a compound which is convertible in vivo by metabolic means (e.g. by hydrolysis) to a compound of any of the formulae herein. Various forms of prodrugs are known in the art, for example, as discussed in Bundgaard, (ed.), Design of Prodrugs, Elsevier (1985); and "Hydrolysis In Drug And Prodrug Metabolism: Chemistry, Biochemistry And Enzymology," John Wiley and Sons, Ltd. (2002).

The invention will be further described in the following examples. It should be understood that these examples are for illustrative purposes only and are not to be construed as limiting this invention in any manner.

All references cited herein, whether in print, electronic, computer readable storage media or other form, are expressly incorporated by reference in their entirety, including but not limited to, abstracts, articles, journals, publications, texts, treatises, technical data sheets, internet web sites, databases, patents, patent applications, and patent publications.

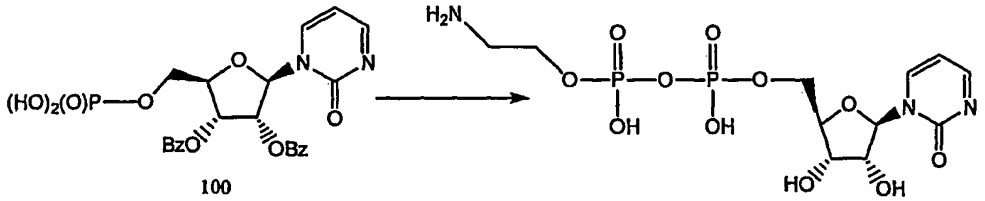
Embodiments are further described in the following representative examples, which do not limit the scope of the invention described in the claims.

Abbreviations: Zeb, zebularine; Zeb-MP, zebularine-5'- monophosphate; Zeb-DP, zebularine-5'-diphosphate; Zeb-TP; zebularine-5'-triphosphate; Zeb-DP-EA, zebularine-5'-diphosphoethanolamine; Zeb-DP-Chol, zebularine- 5'-diphosphocholine; CPEU, cyclopentenyl uridine; IC_{50}, drug concentration resulting in 50% inhibition of growth; PDE-1, snake venom phosphodiesterase-1; AP, alkaline phosphatase; UCK, uridine/cytidine kinase; NCI, US National Cancer Institute.

1. Chemistry

1.1. Zebularine and corresponding 2'-deoxy and 2'-fluoro analogues are known in the literature. See, Driscoll et al., *J. Med. Chem.* 34, 3280-3284 (1991); Barchi et al., *Nucleosides & Nucleotides*, 11, 1781-1793 (1992).

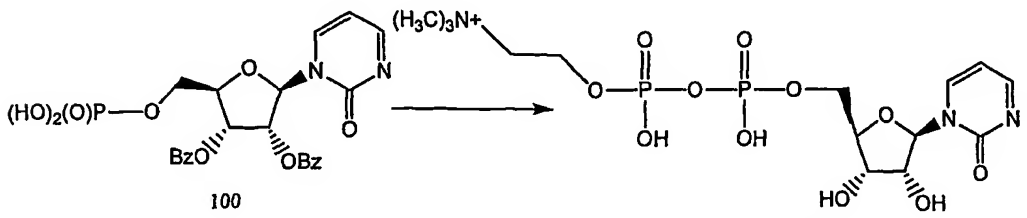
1.2



100 101

Compound 100 is reacted with phosphoric acid mono-[2-(1,3-dioxo-1,3-dihydro-isoindol-2-yl)-ethyl] ester in the presence of DCC in methylene chloride to provide the corresponding diphosphate. The resulting diphosphate is deprotected (hydrazine; methanolic ammonia) to provide compound 101. The 2'-deoxy and 2'-fluoro analogs of compound 101 can be made similarly from available 2'-deoxy and 2'-fluoro analogs of compound 100.

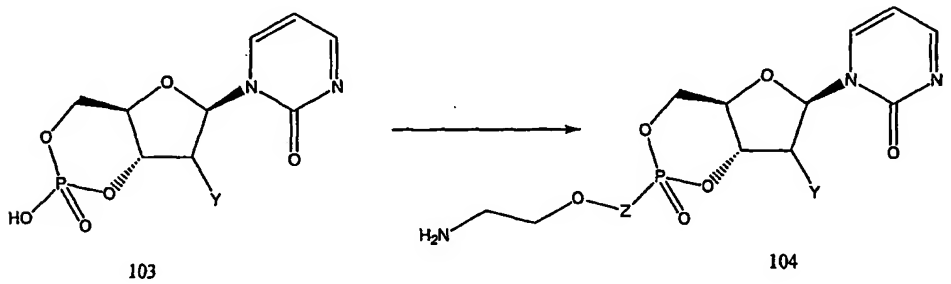
1.3



100 102

1,3-dicyclohexylcarbodiimide (DCC) in methylene chloride to provide the corresponding diphosphate. The diphosphate is hydrolized under methanolic ammonia conditions to provide compound 102. The 2'-deoxy and 2'-fluoro analogs of compound 102 can be made similarly from available 2'-deoxy and 2'-fluoro analogs of compound 102.

1.4



103 104

Y = F

Compound 103 (available via literature procedures) is reacted with 2-phthalimidoethyl bromide in the presence of DCC in methylene chloride. The resulting compound is treated with hydrazine to provide compound 104.

2. Materials and methods

2.1. Chemicals and reagents

Zebularine (1-(β-D-ribofuranosyl)1,2-dihydropyrimidin-2-one, 2(1H)-pyriidinone riboside, NSC 309132) and [2-^{14}C]Zeb (51 mCi/mmol) were obtained from the Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, NCI (Bethesda, MD). [Methyl-^{3}H]choline chloride (80 Ci/mmol) and [1-^{3}H]ethanol-1-ol-2-amine hydrochloride (31 Ci/mmol) were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Choline chloride, ethanolamine HCl, Tri-reagent® kits and selected nucleoside and nucleotide standards were obtained from Sigma Chemical Co. (St. Louis, MO). The enzymes deoxyribonuclease I (DNase I, type II [bovine pancreas], EC 3.1.21.1), ribonuclease A (type I-A, [bovine pancreas], EC 3.1.27.5), PDE-1 (type VII [Crotalus atrox venom], EC 3.1.4.1), and

CA) from parent nucleoside and had an HPLC purity >98%. A Zeb-MP standard was produced by complete enzymatic conversion of Zeb-TP by PDE-1. 2'-Deoxyzebularine [G] and CPEU [F] were synthetic products that were available from previous studies in our laboratories. All other chemicals and reagents were of the highest quality commercially available.

2.2 Cell culture

Human T-24 bladder carcinoma and human Molt-4 lymphoid cells were obtained from the American Type Culture Collection (Rockville, MD). The EJ6 cell line, a tumorigenic derivative of T-24 cells, was kindly provided by Dr. Eric J. Stanbridge, Department of , University of California at Irvine, and murine MC38 colon cancer cells were a gift from Dr. Steven A. Rosenberg, Surgery Branch, Center for Cancer Research, NCI. T-24, EJ6 and MC38 cells were cultured in DMED media while Molt-4 cells were grown in RPMI 1640 media. All media were supplemented with 10% heat-inactivated fetal calf serum, 4 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were in logarithmic growth at the time of use and were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

2.3. Effect of zebularine on T-24 cell growth

Exponentially growing T-24 cells were cultured in 24-well plates (10^5 cells per well) overnight. Cells were then washed with fresh medium and varying concentrations (0-1000 μM) of zebularine were added. After incubation for 48 h, cells were trypsinized, collected and counted in a ZB1 Coulter Counter. The cell growth rate was expressed as a percentage of the increase in cell number of the untreated control cultures. The IC_{50} was calculated from the linear portion of the growth inhibition curve.

2.4. Separation and measurement of zebularine and its cellular metabolites

2.4.1. Preparation of cell extracts

After the appropriate incubations with [2-^{14}C]Zeb, cells were washed three times with PBS, treated with trypsin and collected by centrifugation at 1500 x *g* for 10 min.

removed and heated at 95°C for 2.5 min. The heated extract was centrifuged at 12,000 x *g* for 10 min and the clear supernatant fraction was removed and evaporated to dryness under N_2. This sample was reconstituted in 250 µl H_2O and aliquots were subjected to gradient anion-exchange chromatography as described below.

2.4.2. Gradient anion-exchange HPLC

Separations of Zeb and its phosphorylated metabolites were carried out using a Hewlett-Packard 1100 HPLC system with a diode-array ultraviolet absorption detector and controlled by ChemStation software (Version 6.01). A Whatman Partisil-10 SAX column (250 X 4.6 mm) certified to have greater than 100,000 plates/m was used with the following elution program: 0-5 min, 100% buffer A (0.01 M ammonium phosphate, native pH); 5-20 min, linear gradient to 25% buffer B (0.7 M ammonium phosphate with 10% methanol); 20-30 min, linear gradient to 100% buffer B; 30-40 min, isocratic buffer B; 40-55 min, linear gradient to 100% buffer A and equilibration. The flow rate was 2 ml/min throughout. One-minute fractions were collected and radioactivity was determined by scintillation spectrometry. Fractions containing radiolabeled Zeb-containing nucleotides were quantified based on the known specific activity of [2-^{14}C]Zeb. To confirm that the zebularine base was present in these radiolabeled nucleotides, aliquots of the cell extract were enzymatically treated to degrade all nucleotides to nucleosides and analyzed by anion-exchange and reverse phase HPLC as described more fully below.

2.4.3. Reverse-phase HPLC

Reverse-phase HPLC was employed for the separation and identification of zebularine nucleosides in cell extracts, for measuring [2-^{14}C]Zeb after enzymatic degradation of its phosphorylated metabolites, and for the DNA and RNA studies. Separations were carried out on a 5-µm Beckman Ultrasphere C_{18} column (250 X 4.6 mm) using the following gradient elution program with a flow rate of 2 ml/min throughout: 0-25 min, linear gradient from 1% to 25% methanol; 25-30 min, isocratic with 25% methanol; 30-40 min, linear gradient to 1% methanol, and equilibration. One-minute fractions were collected and radioactivity was determined by liquid

2.5. Enzymatic characterization of zebularine metabolites

Characterization of zebularine metabolites was carried out by selective enzymatic degradation of cellular extracts as previously described [A]. Briefly, the lyophilized methanolic cell extract was dissolved in 100 µl of 0.01 M Tris•HCl, pH 9.0 containing 1 mM $MgCl_2$, and 1.5 U AP or 0.03 U PDE-1 was added to the appropriate aliquots. Samples were incubated for 6 h at 37 °C, enzymes were inactivated by heating at 95 °C for 2.5 min, and aliquots were then analyzed by anion-exchange and C_{18} reverse phase HPLC as described above.

2.6. Biosynthesis of doubly labeled choline and ethanolamine adducts

T-24 cells were cultured in DMEM medium supplemented with 10% FCS and 4 mM L-glutamine but without endogenous choline or ethanolamine. The cells so grown exhibited doubling times (24 h) identical to those of T-24 cells in choline replete medium. To determine whether choline and ethanolamine adducts are formed during zebularine metabolism, T-24 cells were incubated with 100 µM [2-^{14}C]Zeb (1 µCi/ml) alone and in combination with either 28 µM [methyl-^{3}H]choline (10 µCi/ml) or 50 µM [1-^{3}H]ethanolamine (10 µCi/ml) for 24 h in separate parallel experiments. Thereafter, cells were harvested, washed and extracted with 60% methanol. Zebularine metabolites were determined by gradient ion-exchange HPLC as described above.

2.7. Dose-dependent formation of zebularine metabolites

Triplicate aliquots of logarithmically growing T-24 cells (1 X 10^6 cells) were incubated for 6 h with concentrations of [2-^{14}C]Zeb (1 µCi/ml) ranging from 1-500 µM. At the end of the incubations, methanolic extracts were prepared and amounts of zebularine metabolites were determined by gradient anion-exchange HPLC as described before.

2.8. Rates of accumulation and decay of zebularine metabolites

T-24 cells in logarithmic growth were incubated with 10 µM [2-^{14}C]Zeb (1 µCi/ml

metabolites as previously described. After 24 h of exposure to drug, the cells were washed three times with fresh medium and re-incubated in drug-free medium. Aliquots of this incubation mixture were then sampled periodically for the ensuing 24-h period and the same work-up and analysis carried out. Apparent disappearance half-lives after removal of drug were determined for individual metabolites by non-linear least squares analysis of their concentration *versus* time profiles using a monoexponential decay function.

2.9. Effect of cytidine, uridine and CPEU on zebularine phosphorylation

Logarithmically growing T-24 cells (1 X 10^6 cells) were incubated for 6 h with 10 μM [2-^{14}C]Zeb (1 μCi/ml) alone and in combination with either 10 or 50 μM of cytidine, uridine or CPEU, respectively. At the end of incubation, duplicate aliquots of cells were harvested and methanolic extracts were prepared and analyzed as previously described

2.10. Incorporation of zebularine into cellular DNA and RNA

DNA and RNA were isolated using the TRI-reagent® procedure [B,C]. Briefly, 5 X 10^7 T-24 cells were incubated for 24 h with 10 μM [2-^{14}C]Zeb (1 μCi/ml). At the end of the incubation, cells were washed 3 times with cold PBS, harvested by trypsinization and collected by centrifugation. One ml TRI-reagent® (guanidine thiocyanate and phenol in a monophasic solution) was added to the cell pellet, solubilizing the DNA, RNA and protein. Chloroform (0.2 ml) was then added and the mixture was centrifuged at 12,000 X *g* for 15 min. RNA (in the aqueous phase) and DNA (in the interphase) were then separated according to the TRI-reagent® protocol, and the radioactivity in each quantified by liquid scintillation counting.

The isolated DNA and RNA were hydrolyzed overnight at 37 °C in 1 ml of pH 7.4, 0.1 M HEPES buffer containing 140 μg of either DNase or Rnase and 0.02 U PDE-1 and 5 U AP. One hundred-microliter aliquots of this reaction mixture representing approximately 25 μg of hydrolyzed DNA or RNA were analyzed by reverse-phase HPLC as described above to determine the presence and amount of either [2-^{14}C]2'-dZeb (DNA) or [2-^{14}C]Zeb (RNA).

2.11. Metabolism of zebularine in EJ6-derived tumors

All animal care and experiments were performed in accordance with the guidelines of the Animal Care and Use Committee of the Ben-Gurion University of the Negev. Male BABL/c *nu/nu* mice (Harlan, San Diego, CA)(n=6), 6-8 weeks of age, were kept at 22 ± 1° C in 40- 60% relative humidity with alternating 12-h periods of light and dark and maintained on a diet of commercial-pelleted mouse food (Purina Chow) with free access to food and water. Mice were inoculated s.c. with 2.5 X 10^6 EJ6 cells each into both the left and right flanks. After a period of 2-3 weeks during which macroscopic tumors (50-200mm^3) developed, the mice (mean weight = 25 ± 4 gm) were treated i.p. with 500 mg/kg [2-^{14}C]Zeb (500 μCi/ml). EJ6 tumors and straight muscle were removed from mice under ether anesthesia 24 h after drug administration and immediately frozen in liquid N_2 and stored at –70 °C pending sample work-up and analysis. Tumors and muscle tissue (100-200 mg) were homogenized at 4 °C in 1.0 ml of 60% methanol using a Polytron homogenizer. The homogenate was heated at 95°C for 3 min and centrifuged at 12,000 X *g* for 10 min at 4 °C. The supernatant was collected and evaporated under a nitrogen stream. The resulting residue was then dissolved in deionized water and appropriate aliquots subjected to anion-exchange HPLC analysis as described by Noy *et al.* [D].

Zebularine exhibited a moderate cytostatic effect on logarithmically growing T-24 cells in culture. As seen in Fig. 2 and Fig. 10, a 48-hr exposure to the drug was able to inhibit T-24 cell proliferation with an IC_{50} of 145 μM or less. This antiproliferative effect appeared to reach a plateau at concentrations of 500 μM and above for this exposure time.

3.2. Cellular metabolism of zebularine

A requirement for the biological activation of nucleoside analogues is their anabolic conversion to nucleotides. In the case of zebularine, conversion to the triphosphate appears to be a prerequisite for eventual incorporation into DNA and the ability to function as an inhibitor of DNA methylation. Therefore, the ability of zebularine to be phosphorylated was assessed using one murine and two human cell lines. This was accomplished by exposing exponentially growing cells to 10 μM [2-^{3}H]Zeb for 6 h, then extracting the cells with 60% methanol, and subjecting the extract to gradient-elution, ion-exchange chromatography with radiochemical detection. Typical radiochromatograms obtained after zebularine treatment of T-24 cells are shown in Fig. 3 and Fig. 4A. In addition to the parent drug, which elutes close to the void volume (2 min), five acidic metabolites were observed. Three of these metabolites with retention times of 9, 18 and 29 min, respectively, corresponded to the 5'-mono-, di- and triphosphates of zebularine. These assignments were made by comparison with authentic standards and are supported by enzymatic digestion of the extracts with PDE-1 and AP (Fig. 4B and 4C). Treatment of the cellular extract with PDE-1 resulted in the disappearance of the two HPLC peaks corresponding to Zeb-DP and Zeb-TP and produced a concomitant increase in the peak corresponding to Zeb-MP (Fig. 4B). AP digestion of another aliquot of this extract eliminated the HPLC peaks corresponding to Zeb-MP, Zeb-DP and Zeb-TP and generated an increase in parent nucleoside (Fig. 4C).

The remaining two metabolites, eluting at 5 min and 11 min respectively, were identified as phosphodiester conjugates of ethanolamine and choline based on the following evidence. The fact that both metabolites were quantitatively decomposed by PDE-1, with one of the products being Zeb-MP (Fig. 4B), strongly suggested that

was unlikely. To assess whether choline and ethanolamine could be directly utilized by T-24 cells to form Zeb-DP conjugates, T-24 cells were incubated with [2-^{14}C]Zeb and [^{3}H]choline or [^{3}H]ethanolamine. HPLC and radiometric analysis of the cellular extracts resulting from these double-label experiments indicated that the two metabolites eluting with retention times of 5 and 11 min, respectively, did indeed contain both ^{14}C and tritium. When T-24 cells were incubated with [2-^{14}C]Zeb and [^{3}H]ethanolamine, the peak eluting at 5 min contained both labels (Fig. 5B). When [^{3}H]choline was used with [2-^{14}C]Zeb, the double label was associated with the peak with a retention time of 11 min (Fig. 5C). Furthermore, no tritium was associated with either peak when [^{3}H]ethanolamine or [^{3}H]choline was incubated with T-24 cells in the absence of zebularine. Thus the two metabolites eluting at 5 and 11 min can be identified as phosphodiesters of zebularine conjugated with ethanolamine and choline, respectively.

Zebularine phosphorylation was also evaluated in Molt-4 human lymphoblasts and in MC38 murine colon carcinoma under conditions comparable to those employed for T-24 bladder carcinoma. As can be seen in Table 1, the five zebularine metabolites were also observed in these two cell lines after a 6-h incubation with 10 μM [2-^{14}C]Zeb. For this particular time point, levels of each of the five zebularine metabolites were highest in the MC38 cells with the concentration of Zeb-TP being 6-fold greater than in T-24 or Molt-4 cells. Zeb-TP and the Zeb-DP-Chol adduct were the major phosphorylated metabolites in all the cell lines, with the former being highest in Molt-4 and MC38 and the latter greatest in T-24 bladder carcinoma. Individual metabolites levels were comparable in the two human cells lines except for the Zeb-DP-Chol adduct which was 3-fold higher in T-24 cells.

3.3. Dose-dependent formation of zebularine metabolites

The formation of the five zebularine metabolites was evaluated as a function of zebularine dose in T-24 cells. Cells were incubated with increasing concentrations of [2-^{14}C]Zeb (1 - 500 μM) for 6 h, at which time the levels of zebularine metabolites were measured. As can be seen in Fig. 6, levels of all metabolites increased with increasing zebularine dose. The rates of this dose-dependent formation were the

concentrations of these two major metabolites were more than 4-fold greater than the other metabolites and approached or exceeded the nanomole per million cell level. Furthermore, the formation of Zeb-TP did not appear to be saturable in contrast to the other metabolites whose levels start to plateau at 250 μM zebularine (Fig. 6). Thus in the case where the zebularine dose was increased to 500 μM, Zeb-TP levels exceeded those of the Zeb-DP-Chol adduct.

3.4. Rates of accumulation and decay of zebularine metabolites

The 24-h intracellular accumulation of individual zebularine metabolites was evaluated for T-24 cells using a dose of 10 μM [2-^{14}C]Zeb. After incubation with radiolabeled drug for an initial 24-h period, cells were washed three times and reincubated in drug-free media so that the decay rate of these metabolites could be determined over the ensuing 24-h period. As described in Materials and Methods, levels of the various zebularine metabolites were determined at timed intervals over the entire 48-h period. Fig. 7 depicts the concentration *versus* time profiles of the intracellular accumulation and decay of the individual zebularine metabolites. Zeb-MP, Zeb-DP and Zeb-TP all exhibited an initial rapid rate of intracellular accumulation over the initial 4 h before reaching a more or less constant steady-state level by 8-12 h. The rate of accumulation of the phosphodiester adducts was more gradual and sustained, since both the Zeb-DP-EA and Zeb-DP-Chol adducts increased over the entire 24-h period and did not reach a steady-state. Upon removal of parent drug, Zeb-DP and Zeb-TP decayed rapidly with roughly equivalent half-lives of 1.5 h and 1.7 h, respectively. The intracellular disappearance of the phosphodiester adducts was much more gradual with estimated half-lives of 6.1 h for the Zeb-DP-EA adduct and 5.4 h for the Zeb-DP-Chol adduct. In contrast to the zebularine 5'-phosphates, these two metabolites could still be detected in the cellular extract 24 h after removal of drug. The intracellular elimination rate of Zeb-MP was intermediate to that of the other metabolites with a half-life of 4.1 h.

3.5. Effect of cytidine, uridine and CPEU on zebularine phosphorylation

Since zebularine is an analogue of cytidine (Fig. 1), it was of interest to ascertain

determined in T-24 cells incubated with 10 μM [2-^{14}C]Zeb for 6 h in the presence of the natural UCK substrates cytidine and uridine and the UCK inhibitor CPEU. As shown in Table 2, 10 μM cytidine reduced the concentration of all zebularine metabolites except Zeb-DP by more than 50%. Increasing the cytidine concentration to 50 μM led to the almost complete abrogation of zebularine anabolism; Zeb-MP and the two phosphodiester adducts could no longer be detected in the cellular extract. In contrast to cytidine, uridine at either 10 μM or 50 μM had no significant inhibitory effect on zebularine metabolism. Cyclopentenyl uridine, a potent inhibitor of UCK with low cytotoxicity [F], was more effective than cytidine in restricting zebularine phosphorylation at the 10 μM level and at least equally effective at 50 μM. These data strongly suggest that the initial phosphorylation of zebularine in T-24 cells is indeed mediated by UCK.

3.6. Incorporation of zebularine into cellular DNA and RNA

The ability of zebularine to be incorporated into host cell nucleic acids was evaluated by examining DNA and RNA isolates from T-24 cells following 24 - 72 h exposure to 10 μM [2-^{14}C]Zeb. As illustrated in Fig. 8A, the vast majority of incorporated radioactivity was found in the RNA of treated cells. DNA incorporation of radiolabel, while observable and significant, was only on average about 15% that of RNA. Subsequent reverse-phase HPLC analysis of the free nucleosides generated from the complete enzymatic digestion of the isolated DNA and RNA revealed that the radiolabel coeluted with zebularine itself in RNA and with authentic 2'-deoxyzebularine in DNA and that no other radioactive peaks were seen in either case (Fig. 8B). Measurement of radiolabeled zebularine and 2'-deoxyzebularine in the RNA and DNA isolated from T-24 cells treated 10 μM [2-^{14}C]Zeb for 48 and 72 h indicated little change from the levels observed at 24 h.

3.7. Metabolism of zebularine in EJ6-derived tumors

The *in vivo* phosphorylation of zebularine was assessed following an i.p dose of 500 mg/kg [2-^{14}C]Zeb to nude mice bearing EJ6-derived T-24 tumors. Both tumor and normal muscle tissue were examined for the presence of zebularine metabolites

occurred (Fig. 9A). Most of the radioactivity was accounted for by 6 principal peaks, which were identified as parent compound and the five metabolites observed *in vitro* (Zeb-MP, Zed-DP, Zeb-TP and the Zeb-DP-EA and Zeb-DP-Chol adducts). The relative amounts of the various phosphorylated metabolites of zebularine were quite similar to that observed for T-24 cells in culture after drug exposure (Fig. 3 and 4A). In contrast, only a small amount of radioactivity corresponding to phosphorylated metabolites could be detected in a similar extract of normal, straight muscle tissue (Fig. 9B). In this latter case, only limited metabolic conversion was observed, with the major zebularine-related compound being parent drug itself (Table 3).

Table 1

Intracellular levels of zebularine metabolites in human and murine tumor cells after incubation with 10 μM [2-^{14}C]Zeb for 6 h

Metabolite	Concentration[a] (pmole/10^6 cells)		
	T-24	Molt-4	MC38
Zeb-MP	13.8 ∀ 0.84	12.8 ∀ 2.5	30.6 ∀ 2.3
Zeb-DP	10.0 ∀ 0.69	5.2 ∀ 1.8	26.9 ∀ 3.2
Zeb-TP	25.8 ∀ 2.2	22.2 ∀ 4.1	152 ∀ 11
Zeb-DP-EA adduct	1.5 ∀ 2.0	1.1 ∀ 0.2	1.8 ∀ 0.4
Zeb-DP-Chol adduct	57.9 ∀ 2.8	17.4 ∀ 3.0	103 ∀ 8.7

[a]mean ∀ SD from three experiments.

Relative effect of cytidine, uridine and cyclopentenyl uridine on zebularine metabolism in T-24 cells after incubation with 10 μM [2-^{14}C]Zeb for 6 h

	Zeb-MP	Zeb-DP	Zeb-TP	Zeb-DP-EA Adduct	Zeb-DP-Chol Adduct
	(% of Control)[a]				
Control (10 μM Zeb)	100[b]	100	100	100	100
10 μM Cyd + 10 μM Zeb	34	71	49	44	16
50 μM Cyd + 10 μM Zeb	ND[c]	22	11	ND	ND
10 μM Urd + 10 μM Zeb	134	110	113	103	91
50 μM Urd + 10 μM Zeb	100	115	100	66	71
10 μM CPEU + 10 μM Zeb	38	21	29	26	22
50 μM CPEU + 10 μM Zeb	4	3	6	6	ND

[a] Control levels (pmol / 10^6 cells) were: Zeb-MP, 5.1; Zeb-DP, 2.2; Zeb-TP,6.7; Zeb-DP-EA Adduct, 2.4; Zeb-DP-Chol Adduct, 22.
[b] Average of duplicate measurements.
[c] Not detectable.

mice bearing the EJ6 variant of T-24 human bladder carcinoma after i.p. treatment with 500 mg/kg [2-^{14}C]Zeb.

Metabolite	Concentration[a] (pmole/100 mg tissue	
	EJ6 Tumor[b]	Striated Muscle[b]
Zeb	1103 ± 482	2682 ± 255
Zeb-MP	694 ± 239	229 ± 23
Zeb-DP	833 ± 328	24 ± 10
Zeb-TP	1068 ± 187	214 ± 39
Zeb-DP-EA adduct	671 ± 392	22 ± 2
Zeb-DP-Chol adduct	2123 ± 191	286 ± 51

[a]mean ∀ SD from three animals.
[b]tissue samples obtained 24 h after drug treatment.

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Figure Legends

Fig. 1. Chemical structures of zebularine, cytidine and 5-azacytidine. The asterisk indicates the position of the [^{14}C]radiolabel in zebularine.

Fig. 2. Effect of 5-azacytidine (!) and zebularine (") on human bladder carcinoma cell proliferation after treatment for 48 h. Points represent the mean ± SD (n = 6). T24 cell number averaged 4 X 10^5 for control at 48 h. Curves were fit (r^2 > 0.990) to a sigmoidal dose-response function allowing variable slope. Calculated IC_{50}s are indicated on the graph.

Fig. 3. HPLC radiochromatogram of [^{14}C]metabolites arising from incubation of T24 cells with 10 µM [2-^{14}C]Zeb (5 µCi/ml) for 6 h. Methanolic extracts were subjected to ion exchange HPLC as described in Materials and Methods.

Fig. 4. Enzymatic characterization of zebularine metabolites isolated from T24 cells incubated with 10 µM [2-^{14}C]Zeb (1 µCi/ml) for 6 h. Equivalent aliquots of

(C). The peak corresponding to the Zeb-DP-EA adduct is indicated by an asterisk.

Fig. 5. Anion-exchange HPLC radiochromatograms of zebularine metabolites in cells treated with [2-^{14}C]Zeb and with either [^{3}H]choline or [^{3}H]ethanolamine. T24 cells (5 X 10^{6}) were incubated with 100 μM [2-^{14}C]Zeb (1 μCi/ml) for 24 h alone (A) or in combination with either 50 μM [^{3}H]ethanolamine (10 μCi/ml) (B) or 28 μM [^{3}H]choline (10 μCi/ml) (C). Cells were extracted and analyzed for zebularine metabolites as described in Materials and Methods.

Fig. 6. Dose-dependent formation of phosphorylated zebularine metabolites in T24 cells. Cells were incubated with [2-^{14}C]Zeb for 6 h and then extracted and analyzed as described in Materials and Methods. Data are mean ± SD (n = 3).

Fig. 7. Concentration *versus* time profile of zebularine metabolites in T24 cells. Cells were incubated with 10 μM [2-^{14}C]Zeb (1 μCi/ml) for the indicated times before being collected, extracted and analyzed as described in Material and Methods. The vertical dotted line indicates removal of drug-containing medium and replacement with drug-free media. Data points are the average of duplicate measurements. A) Zebularine-5'-phosphates. B) Zebularine-5'-diphosphosocholine and ethanolamine conjugates. Symbols are the same as in Fig. 6.

Fig. 8. Incorporation of zebularine into the DNA and RNA of T24 cells after treatment with 10 μM [2-^{14}C]Zeb (1 μCi/ml). A) DNA and RNA were isolated by the Tri-reagent® procedure and incorporated radioactivity was determined as described in Materials and Methods. Values are the mean ± SD (n = 4). B) Reverse-phase radiochromatograms of DNA (!) and RNA (") from zebularine-treated cells digested to constituent nucleosides. DNA and RNA isolated from cells treated with drug for 24 h were digested and analyzed as described in Materials and Methods. Arrows indicate the retention times of authentic Zeb and 2'-deoxyzebularine.

mice were inoculated s.c. with EJ6 tumor cells, which were allowed to grow for 3 weeks. Mice were then treated i.p. with 500 mg/kg [2-^{14}C]Zeb (500 μCi/kg). Twenty-four hours after treatment, mice were sacrificed and tumors (A) and striated muscle (B) were removed and extracted for analysis.

Fig. 10. Effect of zebularine on human bladder carcinoma cell proliferation after treatment for 48 h. Points represent the mean ∀ SD (n = 6). T-24 cell number averaged 4 X 10^5 for control at 48 h. The dashed arrow indicates the IC_{50} for these conditions.

Fig. 11 Metabolic activation pathway of zebularine.

A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Accordingly, other embodiments are within the scope of the following claims.